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Improved thin-layer chromatographic detection of diethylstilbestrol and zeranol in plasma and tissues isolated with alumina and ion-exchange membrane columns in tandem

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ABSTRACT

Clean-up procedures for the isolation of zeranol and diethylstilbestrol (DES) were modified to reduce the analysis time and to increase the efficiency of purification. Several dyes (Fast Blue BB, Fast Corinth V, Fast Blue RR, Fast Blue B, Fast Red Violet B and Fast Violet B) were evaluated, and their minimum detectabilities were determined. Conditions for non-instrumental, semi-quantitative thin-layer chromatography were optimized. Zeranol and DES in plasma and tissues were determined using modified procedures. Enzyme digestion brought about significant improvement in detectabilities of zeranol and DES in both fortified and incurred plasma, serum and tissues. Minimum detectabilities for zeranol and DES were 25 ppb in fortified plasma and tissues. The amount of incurred zeranol measured in the serum of an experimental cow was increased four times, *i.e.* from 50 to 200 ppb, after protease digestion. Glucuronidase digestion showed an eight-fold increase in detection of incurred zeranol levels in bovine liver eight times. These results suggest that digestion releases zeranol and DES from protein and glucuronide complexes, thereby allowing detection of low levels of zeranol and DES which may not be detectable without digestion. Further modification of the purification with an ion-exchange membrane reduced the analysis time by 25%, and the membranes were regenerated up to ten times without loss of activity, allowing an automated process. This method utilizes inexpensive equipment and avoids use of organic solvent, in this case diethyl ether.

INTRODUCTION

More rapid, sensitive and accurate methods are needed to screen and validate the presence of zeranol, diethylstilbestrol (DES) and zearalenone residues in fluids and tissues of farm animals [1]. In our previous publications [2–5] we have summarized relevant chromatographic and immunoassay techniques for the detection of zeranol and DES in fluids and tissues of farm animals.

TLC methods are rapid, inexpensive, do not require high technical skill and are therefore suitable for screening compounds prior to analysis with instrumental methods. The use of diazonium dyes for visualization of estrogen-like compounds was reported by Wortberg *et al.* [6] and Gunther [7] who used Fast Dark Blue R salt and by Scott *et al.* [8] who used Fast Violet B salt. In a recent publication [4] we summarized the reactions of fifteen diazonium dyes with estrogenic growth promoters and presented an optimized procedure for the use of Fast Corinth V salt.

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Objectives of the present study were: modify the clean-up procedures developed in our laboratory; screen minimum detectabilities of Fast Blue BB, Fast Corinth V, Fast Blue RR, Fast Blue B, Fast Red Violet LB and Fast Violet B; analyze zeranol and DES fortified in plasma and tissue extracts; determine the effects of enzyme treatment on the recovery of zeranol and DES added to plasma and tissues; and, with the modified procedure, determine incurred (residual drug from treated animals) zeranol and DES levels in plasma and liver obtained from experimental cows treated with zeranol or DES.

EXPERIMENTAL[a]

Reagents and equipment

Bio-Rex Membrane (AG 1X8) anion-exchange membrane (25-mm syringe filter) (carbonate form) Poly-Prep disposable columns were purchased from Bio-Rad (Richmond, CA, USA), the solid-phase vacuum extractor from J.T. Baker (Phillipsburg, NJ, USA), diethylstilbestrol, zearalenone, Fast Blue B and Fast Red B salt from Aldrich (Milwaukee, WI, USA), Fast Corinth V salt, Fast Blue BB, Fast Red Violet LB salt, β-glucuronidase (1000 U per vial) and crude protease (from bovine pancreas) from Sigma (St. Louis, MO, USA), high-performance silica gel plates (LHP-KD, linear K, 200 μm, 10 cm × 10 cm) with preconcentration zones from Whatman (Clifton, NJ, USA), Merck's HPTLC plates, Kieselgel 60, 10 cm × 10 cm and channeled 10 cm × 10 cm with preadsorbent zone from EM Separations (Gibbstown, NJ, USA) and double-chamber TLC tank for 10 cm × 10 cm plates and Linomat IV sample applicator from Camag (Wrightsville, NC, USA). Sources of other chemicals, reagents and equipment were described in a previous publication [4].

Plasma and tissue samples with incurred zeranol or DES were obtained from the Midwestern Laboratory of Food Safety Inspection Service of the US Department of Agriculture (St. Louis, MO, USA). Tissues used in the fortification study were obtained in local markets while the plasma was obtained from a local slaughter house.

Sample extraction

Bovine tissues and plasma were extracted as described in our previous studies [2,4]. Bovine tissues were homogenized in a Waring blendor and stored at −20°C. Aliquots of 1 or 2.5 *g* were extracted with 4 ml of acetone–water (95:5, v/v) using a Polytron homogenizer for 5 min, and the sample was centrifuged at 3200 *g* for 10 min decanting the supernatant into a silanized calibrated conical tube. The Polytron tip was rinsed with 4 ml of acetone–water and was put aside for subsequent reextraction of the pellet. After centrifugation, the supernatant phases were pooled, and 2–8 ml extracts were transferred to clean-up columns. Bovine liver was extracted in 4 ml of acetone–water by sonication for 1 min at 75% pulsed power. Samples were centrifuged for 10 min at a minimum speed of 3200 *g*. The supernatant phase was decanted, the pellet was extracted again with 4 ml of solvent and centrifuged, and the supernatants were pooled, adjusting the volume to 8 or 10 ml. The extracts were fortified with zeranol or DES at 12.5, 25, 50 and 100 ppb. Aliquots of 2 or 2.5 ml were applied to clean-up columns.

In our previous study [2], irreversible losses of estrogens were observed when these were added to plasma and tissues prior to extraction. In an effort to increase the recovery of zeranol and DES in fortified tissues, samples were heated in a boiling water bath prior to fortification. Fortified tissues were digested with 1 mg of protease (100 μl of 10 mg/ml in 0.01 *M* phosphate buffer, pH 7.5) or 500 U of β-glucuronidase used in 2-h and 250 U in 16-h digestions. Combinations of both enzymes were also used. Acetone–water (95:5) was added to the digests, which were subsequently homogenized with the Polytron or sonicated for 1 min and centrifuged at 3200 *g* (10 min). The supernatants were decanted and aliquots of 2 or 2.5 ml were applied to the clean-up columns.

[a] Reference to a brand or firm name does not constitute an endorsement by the US Department of Agriculture over others of a similar nature not mentioned.

Bovine plasma (1 ml) was extracted with 8 ml of acetone–water using a vortex shaker for 5 min. Plasma was fortified with zeranol and DES at 12.5, 25, 50 or 100 ppb before and after extraction. The fortified extracts were then loaded on the clean-up column. As in fortified tissues, attempts to increase the recovery of the drugs was carried out by digesting plasma proteins with 1 mg of protease (100 μl of 10 mg/ml in 0.01 *M* phosphate buffer, pH 7.5) per ml of plasma.

Bovine plasma and liver samples containing incurred zeranol and DES were analyzed by predigesting the samples with protease followed by β-glucuronidase or digesting with β-glucuronidase alone. The digests were extracted with acetone–water prior to purification. Protease (0.1 ml) was added to 1 ml of plasma, and was digested overnight at 37°C with an additional 1 ml of phosphate buffer. Acetone–water solvent (6 ml) was added, the mixture was mixed vigorously or sonicated for 30 s (75% pulsed power), centrifuged for 10 min (3200 *g*) and the supernatant was decanted and applied to the clean-up columns. Aliquots (1 g) of incurred liver samples were sonicated in 1 ml of 0.01 *M* phosphate buffer for 1 min at 75% pulsed power. To the homogenized samples, 1 mg of protease or 250 U of β-glucuronidase was added, and the samples were incubated overnight at 37°C. To the protease digests, β-glucuronidase (0.5 ml) was added followed by digestion for 1 or 2 h. Results of these combined enzyme digestion studies were compared with the results of digestion with glucuronidase alone for 2 or 16 h. These digests were extracted with 6 ml of acetone–water solvent (95:5) by mixing vigorously for 5 min in a vortex mixer or sonicating for 1 min. The sample pellet was separated by centrifugation for 10 min at 3200 *g* and the supernatant was adjusted to an 8-ml volume. Aliquots of 2–2.5 ml were purified through alumina–ion-exchange clean-up columns.

Solid-phase clean-up

The Bio-Rex anion-exchange membrane was attached to a 5-ml polypropylene syringe barrel. A PTFE disk (0.45 μm pore size) was placed at the bottom of the barrel to prevent particles from clogging the membrane. A solvent-resistant two-way valve was attached to the membrane outlet and the membrane was washed with three 1-ml portions of 10% aqueous acetic acid and rinsed with four 1-ml portions of water until the effluent was neutral to pH paper or 10% phenolphthalein in ethanol. The membrane was converted to the phosphate form with saturated aqueous trisodium phosphate (pH 13.2) which had been previously purified by recrystallization (twice). The phosphate solution (2 × 2 ml) was passed through the membrane at a gravity flow of 1 ml/min or with the use of a vacuum solid-phase extractor. Excess phosphate was washed off with distilled water, four 2-ml portions or until the effluent was neutral.

A schematic outline of the modified clean-up procedure is shown in Fig. 1. Aliquots of 2–5 ml muscle tissue extract, 2.5 ml liver extract and 8 ml

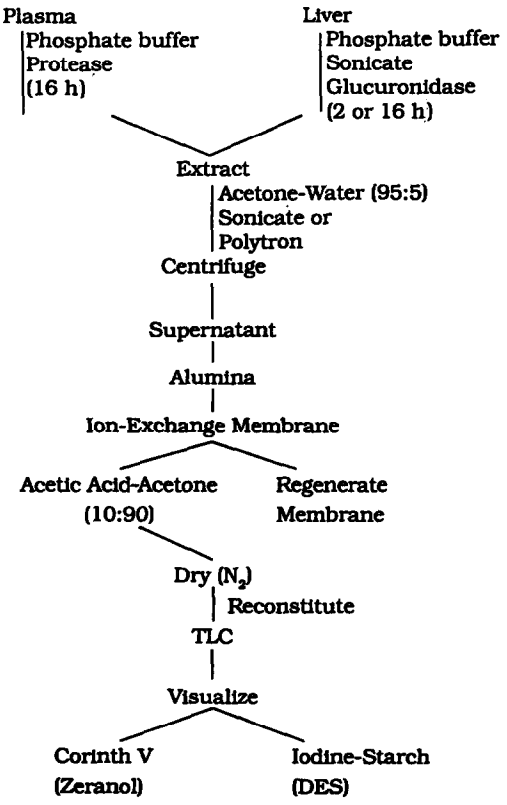

Fig. 1. Modified procedure for the extraction and isolation of estrogens from serum and tissue.

plasma extract were applied to a 1.5-g basic alumina column (packed in Poly-Prep column) placed in tandem with an ion-exchange membrane (in phosphate form) attached to a 5-ml syringe barrel. The ion-exchange membrane and column were placed on a Baker's solid-phase extractor, pulling a vacuum of 2–3 mmHg (7.5–10 kPa). The extracts were allowed to percolate by gravity from the alumina column into the ion-exchange column. After washing the columns with four 1-ml portions of acetone–water (95:5), the alumina column was removed, and the wall of the ion-exchange column was rinsed with 1 ml of acetone–water solvent. The estrogens were then eluted with four 1-ml portions of acetic acid–acetone (10:90, v/v), the effluents being collected in silanized conical glass tubes. Glass tubes were coated with 2% dimethyl–dichlorosilane in toluene, heated at 85°C for 30 min and rinsed with methanol before use. The ion-exchange membranes were regenerated by passing three 1-ml portions of 10% acetic acid in acetone followed by 5% acetone in water and were stored at 4°C. The eluates were dried under a stream of nitrogen. The residues were reconstituted with 100–500 μl of hexane–methanol–2-propanol (80:15:5, v/v). Aliquots of 25, 50 or 100 μl were taken for TLC analysis. Results from the use of membrane for isolation of DES and zeranol were compared with those of the resin, and minimum detectabilities were established.

Preparation of standards

Stock solutions containing 1 mg of zeranol, DES or zearalenone per ml of methanol were prepared and stored below 0°C in silanized glass vials. Dilutions of 10 and 100 μg/ml in methanol were used for fortification. Working dilutions of 0.05–1 μg/ml were prepared with hexane–methanol–2-propanol (80:15:5, v/v) for application onto TLC to keep spots and bands compact.

TLC analysis

TLC plates were immersed horizontally in methanol for 10 min, activated at 85°C for 30 min and stored in a desiccator prior to use. A 10-ml volume of developing solvent (methylene chloride–methanol–2-propanol, 97:1:2, v/v) was placed in each channel of the developing tank and allowed to equilibrate for 15 min. Aliquots of 25–200 μl were applied onto the TLC plates. A Linomat IV sample applicator was utilized for application of 50 or 100 μl onto Merck's 10 cm × 10 cm plates, and 25 or 50 μl were manually applied onto the channeled plates. Samples (50–200 μl) were applied onto Whatman channeled plates. Spots were kept compact with the use of a hair dryer. Plates were placed in the tank and developed for 9 min at room temperature. The developed plates were dried in an 85°C oven or dried with a hair dryer for 5 min and visualized with iodine–starch or diazonium dyes. TLC plates were exposed to iodine vapors for 3–5 min by placing them in a jar saturated with iodine vapor. Background iodine was allowed to volatilize for 30 s before spraying with 0.5% aqueous gelatinized starch solution prepared by heating the starch suspension above 70°C. The starch solution was allowed to cool down before spraying it onto the plate. Alternatively, the developed plates were visualized by spraying with a 0.05% aqueous solution of diazonium dyes which form diazo complexes with zeranol or DES. The plate was placed in a tank saturated with ammonia vapor and exposed for 30 s. The plates were dried further with a hair dryer or heated in an 85°C oven for 2 min until bands appeared.

Initial studies using standard solutions and samples were applied with a Linomat sample applicator at 50 and 100 μl. Merck's channeled plates with preconcentration zones were used for non-instrumental TLC by applying 25- or 50-μl samples. Due to the need of increasing detectability, applying larger sample volumes (50–200 μl), Whatman channeled plates were used in the analysis of fortified and incurred plasma, serum and tissue samples.

RESULTS AND DISCUSSION

Strongly reactive diazonium dyes which form diazo-colored complexes with phenolic and resorcylic groups of estrogenic growth promoters were selected from our previously reported study

[4]. The chemical composition of these dyes and their chemical interactions with anabolic estrogens were also described. Eight diazonium dyes were selected for this study and further screened for their interaction with zeranol. The intensity of the chromogens formed with these dyes and 2.5 ng of zeranol after TLC development was studied. Fast Blue BB and Fast Corinth V formed a stronger color intensity (dark purplish–red) than Fast Blue RR, Fast Blue B, Fast Red Violet LB and Fast Violet B (medium color intensity). Fast Corinth V was used in this study. Although Fast Blue BB can be used interchangeably with Fast Corinth V, the former showed minimal background interference and was preferred for use with a densitometric scanner for quantitative analysis. Iodine–starch visualization was compared with the use of diazonium dyes. Our results show that the use of diazonium dyes resulted in better selectivity and sensitivity for zeranol and zearalenone, whereas DES was better detected with iodine–starch. Visualization of DES with iodine–starch was more sensitive than fluorescence activation [9].

Diethylstilbestrol

DES was analyzed with the modified procedure and detected with iodine–starch. Visualization of DES with iodine–starch was compared with fluorescence activation with 10% sulfuric acid in methanol followed by heating at 90°C for 10 min [9] and viewing at 366-nm ultraviolet light. The TLC plate was also dipped in hexane–ethanol–sulfuric acid (64:35:1) and heated for 45 min at 85°C [10]. DES was detectable at 50 ng (50 ppb) with fluorescence activation and at 25 ng (25 ppb) with iodine–starch visualization. Our previous study [3] also showed that iodine–starch visualization was more sensitive than fluorescence activation with 5% sulfuric acid in ethanol [10]. Attempts were made to stabilize starch suspension and enhance iodine–starch signals with the addition of sodium azide (0.05%) and potasium iodide (1%) to the starch suspension. The resulting TLC plate had a high interfering background which made detection difficult. Spraying of the TLC plate gave better results than immersion. In subsequent analysis of fortified and incurred samples, DES was visualized by exposure to iodine vapor and was sprayed with 0.05% gelatinized starch.

Minimum detectabilities were established by passing DES reference standards (in acetone–water) and fortified extracts of liver and plasma

TABLE I

DETECTION OF DES IN FORTIFIED LIVER EXTRACT USING VISUALIZATION WITH STARCH–IODINE

ND = non-detectable; VF = very faint; F = faint; M = medium; D = dark.

DES standards	0 ng 0 ppb	12.5 ng 25 ppb	25 ng 50 ppb	50 ng on TLC 100 ppb	
Merck	ND	M	M	D	
	ND	VF	M	D	
Whatman	ND	F,F	M,M	D,D	
	ND	F,F	M,M	D,D	
Extracts + DES	0 ppb	25 ppb	50 ppb	100 ppb added	Standard (25 ppb)
Merck	ND	VF	M	D	
	ND	VF	M	D	
	ND	F	M	D	
Whatman (100 μl on TLC)	ND	M	M	D	M
Whatman (50 μl on TLC)	ND	F	F		F

TABLE II

DETECTION OF DES FORTIFIED IN COOKED AND UNCOOKED CALF LIVER

A 1-g amount of homogenized liver was fortified with 50 ppb of DES. ND = non-detectable; VF = very faint; F = faint; M = medium; D = dark.

Standard 50 ng	0; DES	Extract; +DES	+DES; extract	Cooked; extract; +DES	Cooked; +DES; extract	Column blank
D	ND	M	VF	M	M	ND
D	ND	M	VF	M	M	ND

through the clean-up columns. The results of the fortified samples are shown in Tables I-III. Table I shows that the minimum detectability of standards was 12.5 ng in a 25-ppb concentration applied to clean-up columns. In beef liver extracts fortified with 25, 50 or 100 ppb DES and purified through an alumina–ion-exchange column, DES was faintly detectable at 25 ppb and clearly detectable at 50 ppb. These results show that DES was quantitatively recovered in fortified extracts. In our previous studies, fortified extracts were utilized to optimize the purification of estrogenic anabolic compounds through alumina–ion-exchange columns due to irreversible losses of the estrogens when added directly to plasma and tissues. When muscle, liver and kidney tissues were fortified with DES prior to extraction and purification, we observed a lower detectability of DES. However, when muscle, liver and kidney extracts were fortified prior to purification, DES was detected quantitatively. Heat denaturation of proteins can inhibit the binding ability of proteins, and therefore samples were heated prior to fortification with DES to demonstrate the inhibition of protein binding with estrogenic compounds. Table II shows the results of the recovery of DES in fortified cooked and uncooked liver in comparison with the results in fortified extracts (control). Fortification of cooked liver tissue prior to extraction resulted in a quantitative recovery, with these results similar to those of control samples (tissue extracts fortified prior to purification). Fortification of extracts from either cooked or uncooked liver resulted in equally detectable DES levels with medium color intensity of DES bands. These results indicate binding of

TABLE III

ANALYSIS OF PLASMA OR PLASMA EXTRACT FORTIFIED WITH DES

ND = non-detectable; VF = very faint; F = faint; M = medium; D = dark.

	0 ng 0 ppb	12.5 ng 25 ppb	25 ng 50 ppb	50 ng on TLC 100 ppb	
DES standard	ND	VF	M	D	
Fortification level	12.5 ppb	25 ppb	50 ppb	100 ppb added	Standard (100 ppb)
Plasma plus acetone, centrifuged, DES added, purified	ND	ND	VF	M	D
Plasma plus acetone, fortified with DES, centrifuged, purified		ND	ND	F	D

TABLE IV

PROTEASE TREATMENT OF PLASMA FORTIFIED WITH DES

DES added at 50 ppb. ND = non-detectable; VF = very faint; F = faint; M = medium; D = dark.

Standard 50 ppb	0; DES	+ DES; + enzyme digest extract (1 ×)	+ DES; + enzyme digest extract (2 ×)	+ Enzyme digest extract; + DES
D	ND	F	M	D
D	ND	F	M	D

DES to proteins or enzymatic degradation of DES in uncooked liver. Heat denaturation of proteins in tissues inhibited these reactions allowing the quantitative recovery of fortified DES in cooked liver.

Analysis of bovine plasma fortified with DES (Table III) showed results similar to those in fortified liver (see Table IV). DES standards in acetone–water passed through clean-up columns were detectable from 25 to 100 ppb with increasing band intensity (Table III). DES added to plasma extract was detectable at 50 and 100 ppb (Table III). When DES was added to plasma containing acetone–water extraction solvent and then purified, losses were shown with no detectable DES at 25 and 50 ppb (Table III). Losses suggest that DES may have been bound or entrapped in protein precipitates. These results led to the study of the effects of protease digestion of the plasma (Table IV). Results improved when DES (50 ppb) was added to plasma followed by protease addition (100 μl of 10 mg/ml), digested overnight, extracted, purified and analyzed by TLC. The resulting DES band was lighter than the bands from plasma extracts fortified prior to purification. Extraction of digest with two 4-ml portions of acetone–water solvent resulted in bands equivalent to those of a standard control. Our previous work had shown the instability of DES in solution when stored at 4°C [2], breaking down into two bands which were apparently due to isomerization of *trans*-DES to *cis*-DES. Due to this isomerization, the minimum detectability of DES may vary but *cis*-DES is detectable as a faint band. In this study, the mean (± S.D.) R_F values for *trans*-DES and *cis*-DES were 0.33 ± 0.028 (n = 18) and 0.18 ± 0.016 (n = 7), respectively. These R_F values are higher than the previously reported [2] mean R_F values of 0.26 (*trans*-DES) and 0.15 (*cis*-DES) in which case the plates were developed at 4°C compared with room temperature in the present study.

DES was analyzed from incurred tissues after digestion with a combination of protease (10 mg) and β-glucuronidase (500 U) or glucuronidase alone (250 U for 16 h or 500 U for 2 h). The 16-h digestion of liver samples with β-glucuronidase showed the highest extraction of DES with results ≫ 125 ppb (Table V). Protease digestion did not enhance the extraction of zeranol and therefore was not considered for DES extraction in liver. These results demonstrate that DES must be freed from glucuronide complex for quantitative recovery of these compounds.

Zeranol

Zeranol was also purified with the modified procedure and detected with Fast Corinth V or Fast Blue BB. Diazotization of zeranol with Fast Corinth V resulted in better detectability (*i.e.* slightly stronger bands) than with Fast Blue BB when the diazo complex was visually analyzed. Fast Blue BB can be utilized in an instrumental analysis using a densitometric scanner due to minimal background interference compared to Fast Corinth V.

The minimum amount of zeranol standard that was detectable when 50 μl of 100 ppb were directly applied onto a TLC plate was 5 ng, but 12.5 ng were faintly detectable after the purifica-

TABLE V

TLC ESTIMATES OF INCURRED DES AND ZERANOL IN PLASMA AND LIVER

Whatman TLC plates with preconcentration zones were used. Zeranol in serum and liver were visualized with Fast Blue BB and Fast Corinth V, respectively. DES in liver was visualized with starch–iodine. Italic results show the highest recovery. NA = not analyzed.

Treatment	DES in liver (ppb)	Zeranol (ppb)	
		Serum	Liver
No enzyme	NA	50	<50
Protease for 16 h	NA	*200*, 175, 175	<50
Protease for 16 h plus glucoronidase for 1 h	25	NA	<400
Protease for 16 h plus glucoronidase for 2 h	62	NA	*>400*
Glucuronidase for 2 h	>125	NA	*>400*
Glucuronidase for 16 h	≫125	NA	>400

tion step. Zeranol in plasma fortified at 25, 50, and 100 ppb, extracted and purified was not detectable at 25 ppb but faintly detectable at 50 ppb. A summary of the results with fortified plasma is shown in Table VI. The zeranol band intensity from plasma treated with protease and zeranol, digested for 16 h, extracted and then purified was similar to the intensity when plasma was first digested with protease for 16 h, then fortified, extracted and purified (Table VI). Recoveries were quantitative at 25 and 50 ppb. These results agree with those of control plasma extract which was fortified with zeranol prior to purification (Table VI). These results suggest the presence of proteins which bound irreversibly with zeranol and binding was inhibited with the digestion process. With TLC plates from Merck, the mean (± S.D.) R_F of ten analyses of zeranol was 0.22 ± 0.035 when the plate was developed at room temperature for 9 min. The mean (± S.D.) R_F of zeranol when plates were developed at 4°C was 0.17 ± 0.018. In recent studies, using Whatman plates and development at room temperature for 11 min, the mean (± S.D.) R_F in 35 analyses was 0.31 ± 0.013. These results suggest that R_F values must be established in each laboratory or when experimental conditions are changed.

Bovine serum with incurred zeranol was analyzed with the modified procedure. Enzyme digestion of plasma for 16 h increased the amounts of zeranol detected from 50 ppb with no enzyme treatment to 200 ppb with protease digestion. Analysis was made on a plasma sample two years after the initial analysis: approximately 175 ppb of zeranol could be detected. Bovine liver con-

TABLE VI

PROTEASE TREATMENT OF PLASMA FORTIFIED WITH ZERANOL

ND = non-detectable; VF = very faint; F = faint; M = medium; D = dark.

Treatment	25 ppb added	50 ppb added	100 ppb added	Standard (100 ppb)
Plasma, add zeranol, extract, purify	ND	F	M	
Plasma, add protease, digest, add zeranol, extract, purify	F	M	M	D
Plasma, add protease, zeranol, digest, extract, purify	F	M	M	D
Plasma, extract, add zeranol (control)	F	M	D	D

taining incurred zeranol was analyzed with and without enzymes. The results are summarized in Table V. The combined digestion with protease and glucuronidase or glucuronidase digestion alone showed a more than eight-fold increase in measured zeranol. The tolerance level for zeranol in liver was 300 ppb and there was no defined tolerance level for zeranol in serum.

CONCLUSIONS

Significant improvements were made with extraction, isolation and TLC analysis of zeranol and DES in plasma and tissues. The use of an ion-exchange membrane reduced the analysis time by 25% through elimination of water–diethylether extraction and centrifugation steps which were necessary when AGMP-1 resin was used. The membrane was regenerated ten times without loss of activity which makes automation possible. Visualization with Fast Corinth V and Fast Blue BB resulted in highest sensitivity for detection of zeranol and was higher than iodine–starch or fluorescence activation with sulfuric acid. Fast Blue BB had minimal background interference and was therefore suitable for quantitative scanning with a densitometer. DES was best detected with iodine–starch which gave higher signals than derivatization with diazonium dyes and fluorescence activation. Minimum detectabilities for zeranol and DES were 25 ppb (limit of detection 12.5 ng) in fortified tissue extracts but lower signals (lighter TLC bands) resulted when tissues were fortified prior to extraction and purification. Protease digestion of plasma or serum overnight increased the detectability of DES and zeranol in fortified and incurred samples. Detection of DES and zeranol was increased with glucuronidase digestion of fortified and incurred liver samples for 2 h or overnight (16 h). Overnight digestion was carried out with 50% of the glucuronidase concentration used in the 2-h digestion. Use of channeled TLC plates with preconcentration zones increased the minimum detectability from 100 to 25 ppb. This modified procedure provides a rapid and improved method for detection of zeranol and DES. The tolerance level for zeranol in bovine liver tissues is 300 ppb. DES is illegal for use in farm animals and therefore tolerance is zero and this method provides a relatively simple and rapid method to screen for the presence of DES. Furthermore, this modified procedure can also be used for multi-residue analysis of estrogenic anabolic hormones as long as appropriate reference standards are utilized.

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